RESEARCH

Transcriptional activity of oestrogen receptors in the course of embryo development

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Abstract

Oestrogens are well-known proliferation and differentiation factors that play an essential role in the correct development of sex-related organs and behaviour in mammals. With the use of the ERE-Luc reporter mouse model, we show herein that throughout mouse development, oestrogen receptors (ERs) are active starting from day 12 post conception. Most interestingly, we show that prenatal luciferase expression in each organ is proportionally different in relation to the germ layer of the origin. The luciferase content is highest in ectoderm-derived organs (such as brain and skin) and is lowest in endoderm-derived organs (such as liver, lung, thymus and intestine). Consistent with the testosterone surge occurring in male mice at the end of pregnancy, in the first 2 days after birth, we observed a significant increase in the luciferase content in several organs, including the liver, bone, gonads and hindbrain. The results of the present study show a widespread transcriptional activity of ERs in developing embryos, pointing to the potential contribution of these receptors in the development of non-reproductive as well as reproductive organs. Consequently, the findings reported here might be relevant in explaining the significant differences in male and female physiopathology reported by a growing number of studies and may underline the necessity for more systematic analyses aimed at the identification of the prenatal effects of drugs interfering with ER signalling, such as aromatase inhibitors or endocrine disrupter chemicals.

Key Words
- oestrogen receptors
- embryo development
- transcriptional activity
- sex differences
- reporter mice

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Introduction

Oestrogens have been long known as cell proliferation and differentiation factors (Tsai & O'Malley 1994), and several lines of evidence suggest that, in the course of foetal programming, these hormones are relevant for the sexual differentiation of reproductive tissues, including the brain (Phoenix *et al.* 1959, Pang *et al.* 1979, Tobet *et al.* 1986, Korach *et al.* 1988, Greco *et al.* 1991, Rissman *et al.* 1997, Nielsen *et al.* 2000, Albrecht *et al.* 2009). Very little is known regarding the effects of these steroids in non-reproductive organs, in spite of the fact that preclinical and clinical investigations in subjects with prenatal impairment of ER signalling showed physiological alterations and increased incidence of diseases involving the cardiovascular (Conte *et al.* 1994, Jones *et al.* 2000, Tait *et al.* 2015, Yuchi *et al.* 2015), metabolic (Lapid *et al.* 2014), immune (Zoller & Kersh 2006), respiratory (Thuresson-Klein *et al.* 1985), and skeletal systems as well as the epidermis (Hanley *et al.*

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1996, Brandenberger *et al.* 1997, Lemmen *et al.* 1999, Takeyama *et al.* 2001).

The biological effects of the steroid hormone 17β-oestradiol (E2) – the most expressed oestrogen – are predominantly mediated through two distinct ERs (ERα and ERβ) that share a common phylogenetic origin, conserved structural organization and similar mode of action (mainly as ligand-operated transcription factors) with the other members of the nuclear receptor (NR) family (Tsai & O'Malley 1994, Kininis & Kraus 2008). In addition, ERs may interfere with the signalling of other membrane receptors as well as intracellular receptors, and ERα, may associate with the plasma membrane and may activate non-nuclear signalling from this site. These rapid, non-genomic/membrane initiated steroid signals have been characterized in endothelial cells but may be present in other cellular systems (Arnal *et al.* 2017). The functional activation of intracellular ERs occurs through binding with oestrogens, but unliganded ER activation may be triggered by enzymatic activities, inducing post-translational modifications (Dahlman-Wright *et al.* 2006) that enable ER to release inhibitory proteins generally associated with inactive receptor proteins. ERs were the first NRs to be described (Toft & Gorski 1966, Jensen 2005), and they appear to be the closest to the ancestral steroid receptor (Thornton *et al.* 2003). In addition to these intracellular proteins, a membrane receptor, G protein-coupled oestrogen receptor 1 (GPER1), may transduce oestrogen signals through non-genomic signalling (Revankar *et al.* 2005).

In adult mammals, it is well known that ERs are expressed and functionally active in most reproductive and non-reproductive tissue cells (Ciocca & Roig 1995, Maggi *et al.* 2004, Bookout *et al.* 2006). Their expression and transcriptional activity in the course of embryo development is less studied (Brandenberger *et al.* 1997, Lemmen *et al.* 1999). In addition to selective KO mutation of ERα, ERβ or the aromatase gene (*Cyp19a1*, encoding the enzyme responsible for testosterone-derived oestrogen synthesis) that highlighted the relevance of these receptors and their cognate ligands for the development of sexual organs and sexual behaviour (Kudwa *et al.* 2006), the elucidation of the ER distribution and activity during implantation and embryogenesis in non-reproductive tissues is circumscribed to only a few studies (Bondesson *et al.* 2015, Mogi *et al.* 2015, Park *et al.* 2017). However, preclinical and clinical observations in subjects carrying mutations that impair ER signalling showed deviations from the proper development of the cardiovascular system (Del Principe *et al.* 2015, Tait *et al.* 2015), innate immune and neuro-immune communications (Zoller & Kersh 2006), pancreatic and gastric activity (Campbell-Thompson *et al.* 2001, Maniu *et al.* 2016) and liver functions (Bryzgalova *et al.* 2006, Foryst-Ludwig *et al.* 2008, Barros & Gustafsson 2011), as well as adipose (Barros & Gustafsson 2011, Lapid *et al.* 2014), lung (Thuresson-Klein *et al.* 1985, Patrone *et al.* 2003, Carey *et al.* 2007), kidney (Lane 2008, Kummer *et al.* 2011) and epidermal tissues with the muscle-skeletal apparatus (Hanley *et al.* 1996, Brandenberger *et al.* 1997, Lemmen *et al.* 1999, Takeyama *et al.* 2001, Walker & Korach 2004, Markiewicz *et al.* 2013, Ueberschlag-Pitiot *et al.* 2017).

In view of the growing number of reports pointing to significant sex-related differences in mammalian physiopathology and the increasing concern of the potential long-term effects of maternal exposure to EDCs and to drugs (such as aromatase inhibitors) that may interfere with oestrogen signalling during pregnancy, a better understanding of the oestrogen-dependent programmes in ontogeny is indispensable for the comprehension of the role of sex in the incidence of several pathologies and for the creation of efficacious protocols for the evaluation of EDCs exposure.

To verify the extent of ER transcriptional activity during embryogenesis, we investigated the luciferase expression in the ERE-Luc reporter mouse (Ciana *et al.* 2001, 2003). In this mouse, the general transcription of the firefly luciferase transgene is controlled by the activated intracellular ERs. In the last 15 years, a large number of studies carried out in different laboratories showed that, in the ERE-Luc mouse, the amount of luciferase synthesized is proportional to the ER transcriptional activity and quantifiable *in vivo* by bioluminescence imaging or *ex vivo* by means of an enzymatic assay in tissue lysates (Klotz *et al.* 2002, Patrone *et al.* 2003, Humpel *et al.* 2005, Mussi *et al.* 2006, Chambliss *et al.* 2010, Penza *et al.* 2011, Vantaggiato *et al.* 2016). These studies demonstrated (i) a lack of interference of signals originating by the chromatin surrounding the transgene granted by the combination of the specific integration site and insulator sequences flanking the reporter (Rizzi *et al.* 2017), (ii) the selective response to ERs of the synthetic promoter driving luciferase transcription and (iii) that the reporter is transcriptionally viable in all tissues (as also indicated by a background reporter activity due to the TK minimal promoter).

By demonstrating that ERs are widely active during embryo development, our results will facilitate the understanding of complex functions of ERs in embryo maturation, putting novel bases for the comprehension of

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the involvement of sex in the incidence and progression of pathologies not strictly associated with reproductive functions. The data presented may also help with the prediction of the effects of exposure to EDCs or the administration of drugs, such as aromatase inhibitors to pregnant mothers. Therefore, the ERE-Luc reporter mice may be considered a valuable tool to unravel the effects of potential EDCs or drugs administered to pregnant mothers that can interfere with the oestrogen signalling of the foetus.

Materials and methods

Animals

The mice were housed with *ad libitum* access to standard chow (RF21, Mucedola, Italy) and water. We generated heterozygous ERE-Luc foetuses by caging homozygous ERE-Luc males with WT C57/Bl6j females for the night. The day after overnight mating was counted as 0.5 dpc (day post conception). Natural birth occurred on 19.5 dpc, which was counted as day 0 post birth (P0). In the experiments with the knockouts, the mice heterozygous for aromatase (Ar+/−) from E. Simpson were crossed with ERE-Luc mice, and the progeny were as backcrossed to obtain both KO and WT foetuses in the same litter that were genotyped from tail-derived DNA by PCR using published primers (Fisher *et al.* 1998). The sex was confirmed by PCR of the SMC locus (Agulnik *et al.* 1997). All the animal experiments were carried out in accordance with the European Guidelines for human animal care. The use of experimental animals was approved by the Italian Ministry of Research and University and was controlled by the panel of experts of the Department of Pharmacological and Biomolecular Sciences at the University of Milan.

Treatment with ER antagonist

ICI 182,780 powder (Sigma) was dissolved in 99% v/v ethanol to a concentration of 30 mg/mL. A total of 75 µg or 30 µg of ICI, dispersed in 100 µL of maize oil, was s.c. injected 24 h and 6 h before the imaging session, resulting in 1 mg/kg and 2.5 mg/kg for a 33 g female at day ~16 of pregnancy.

In vivo bioluminescence imaging

Pregnant females were anaesthetized with 50 µL s.c. injection of a water solution of 78% ketamine (Ketavet 50 mg/mL, Intervet, Peschiera Borromeo, Italy) and 15% xylazine (Rompun 20 mg/mL, Bayer, Leverkusen, Germany). Once anaesthetized, the pregnant females were shaved to allow a better measurement of the photons emitted from the foetuses. Fifteen minutes before BLI, 90 µL of a water solution of the luciferase substrate luciferin (Beetle luciferin potassium salt, Promega) was administered s.c. (75 mg/kg). Bioluminescence was measured by a Night Owl imaging unit (Berthold Technologies, Bad Wildbad, Germany); the mice were placed in the light-tight chamber, and their pictures were first taken with dimmed light, and then the luciferase signal was registered for 5 min. Merging of the images enabled to visualizing body areas where photon emission occurred (luciferase signal was transformed in pseudocolours: blue-lowest, then green, red, yellow and white as the highest signal). For quantification, photon emission was measured in the whole body areas (counts per second, cts/s). Normalization was performed using an external source of photons (Glowell, Lux Biotechnology, Edinburgh, UK) enabling measurement of the instrumental efficiency of photon counting. All the measurements were performed using WinLight32 (Berthold Technologies). After BLI, the foetuses were dissected under binocular macroscopy, and the organs shown in the figures were collected, frozen on dry-ice and stored at −80°C until assayed.

Luciferase enzymatic assay

The tissues were homogenized in 200 µL ice-cold lysis buffer (100 mM KPO_4, 1 mM DTT, 4 mM EGTA, 4 mM EDTA, pH 7.8) with a micro pestle (Eppendorf); then, they underwent freezing and were thawed during centrifugation at 4900 ***g***, 4°C for 25 min. The supernatants were collected, and the protein concentrations were measured by Bradford assay following the manufacturer's instructions (Pierce Biotech). Luciferase enzymatic activity was measured with a commercial kit (Luciferase Assay System, Promega) in a luminometer (Glomax, Promega) and was expressed as the relative light units over 10 s/µg protein (RLU/µg proteins).

Immunohistochemistry

The mice at 17.5 dpc were fixed in 4% paraformaldehyde, embedded in paraffin and whole-sliced. Sections of 5 µm were deparaffinized in xylene and rehydrated in decreasing ethanol concentrations. After washing, the slides were microwaved in citrate buffer, washed in PBS and incubated 15 min in 1% H_2O_2.

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For luciferase detection: After three PBS washes, the sections were permeabilized in 0.2% Triton-X-100 in PBS and blocked by incubation with 10% goat serum and 0.3% Tween 20 in PBS for 30 min at 37°C, followed by biotin blocking buffer (Vector Laboratories). The sections were incubated in a humidity chamber overnight at 4°C with rabbit anti-luciferase antibody or normal rabbit serum diluted 1:6000 in blocking buffer. The anti-luciferase antibody was provided by W Just (Soto *et al.* 1993). Next, the sections were washed in 0.3% Tween 20 PBS and were incubated at room temperature for 60 min with biotinylated goat anti-rabbit IgG (Vector Laboratories) and diluted 1:200 in 1% normal goat serum 0.3% Tween 20. Detection was performed with the Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions. The sections were counterstained with haematoxylin/eosin.

For ERα detection: After three PBS washes, the sections were permeabilized and blocked by incubation with 10% goat serum, 0.3% Triton-X in PBS for 120 min at RT, followed by biotin blocking buffer (Vector Laboratories). The sections were then incubated in a humidity chamber overnight at 4°C with anti-ERα antibody (polyclonal, made in rabbit, Abcam ab75635) or normal rabbit serum was diluted 1:100 in blocking buffer. Next, the sections were washed in 0.3% Tween 20 PBS and were incubated at room temperature for 60 min with biotinylated goat anti-rabbit IgG (Vector Laboratories), diluted 1:200 in 1% normal goat serum. Detection was performed with the Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions.

Statistical analysis

P values were calculated as described in the figure legends using GraphPad Prism version 5.01 for Windows, GraphPad Software.

Results

The *in vivo* BLI of WT female mice crossed with homozygote male ERE-Luc clearly showed luciferase activity in living foetuses growing *in utero*. Figure 1A shows that by exposing the pregnant mothers to CCD, photon emission from the foetuses was measurable from 14.5 days post conception (dpc), increasing in the course of pregnancy and was highest at 18.5 dpc. Considering that the mother tissues shielded some of the embryo bioluminescence, to obtain better insight into the onset of luciferase synthesis in the embryos, we investigated the BLI pattern of ER activity in excised embryos.

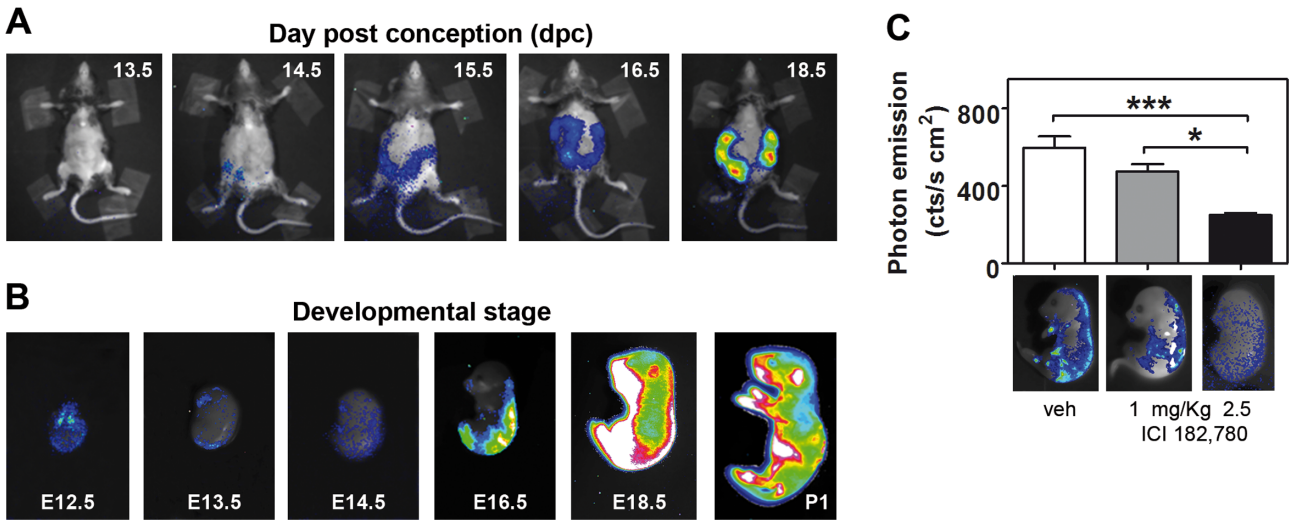

Figure 1
ER is transcriptionally active in the developing mouse. (A) *In utero* imaging: anaesthetized WT pregnant mothers carrying ERE-Luc foetuses were injected with the substrate luciferin 30 min prior to imaging, and photon emission was recorded in BLI units. Merging of the pseudo colour-transformed signal with the pregnant picture allows the identification of transcriptional active oestrogen receptors in the developing litter (blue: low, white: high). The mothers were shaved to improve photon imaging. (B) Representative BLI taken in living foetuses immediately after uterine excision and of a newborn mouse at P1. The sex of the mouse at P1 is male. (C) 24 h and 6 h before litter collection, pregnant females were treated with vehicle or 1 mg/kg or 2.5 mg/kg of the ER pure antagonist ICI 182,780. Foetuses were excised at 16.5 dpc and were subjected to BLI. Photon emission (cts/cm^{2}s) was measured in the whole (upper) foetal area and was transformed in a pseudocolour image merged on the mice pictures (lower). Bars represent the mean ± S.E.M. *P<0.05 calculated with one-way ANOVA followed by Bonferroni *post hoc* test.

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Therefore, the mothers were injected with luciferin and killed for the dissection and rapid measurement of BLI in each individual embryo (Fig. 1B). Photon emission was measurable starting at 12.5 dpc but not before. At 12.5 dpc, photon emission was quite diffuse in the whole embryo, with higher emission in the telencephalon, in the proximity of the eyes, in the heart and in the spinal ganglia. The finding of luciferase activity at 12.5 dpc was consistent with gonad differentiation from a bipotential to sexually differentiated state (as indicated by transcriptome analysis) (Munger *et al.* 2013) and with the highest foetal production of oestrogens (Lemmen *et al.* 2002). At 13.5 dpc, photon emission was more circumscribed to the brain and peripheral nervous system. From 16.5 dpc to 18.5 dpc, luciferase emission increased from the dorsal to the ventral skin, and one day post birth (P1), the signal was maximal in the hepatic/abdomen area and in the limbs.

To rule out that oestrogen-related receptors (ERRs), which are widely expressed in embryos (Bonnelye *et al.* 1997, Luo *et al.* 1997), were active on the promoter of the ERE-Luc mouse, we treated pregnant females with the ER pan-antagonist fulvestrant (ICI 182,780), which has been reported to be an agonist of ERRs (Li *et al.* 2010). Treatment of the mothers with 1 mg/kg and 2.5 mg/kg ICI 182,780 was associated with a dose-dependent decrease in photon emission in foetuses (Fig. 1C), pointing to the absence of significant contributions from ERRs to luciferase synthesis. To further demonstrate the strict association between photon emission and ER-dependent luciferase expression, mice at 17.5 dpc were subjected to BLI and were subsequently whole-mounted for staining with anti-luciferase antibodies. The intensity and distribution of the peroxidase staining clearly reproduced the BLI (Fig. 2A and B) and enabled the definition of the cells expressing the reporter enzyme at the cellular level. The specificity of the luciferase staining was tested in several tissues of ERE-Luc and WT mice (Fig. 2C, D, E, F, G, H and I) and, importantly, the immuno-decoration of luciferase reproduced the staining obtained with anti-ERα antibodies in several tissues (whole snout, muscle, bone, whiskers, skin) (Fig. 2L, M, N, O and P).

This experiment, together with previous BLI observations, was a clear indication of the widespread ER transcriptional activity. In addition to brain and reproductive organs, we found significant amounts of luciferase activity in several non-reproductive tissues. The overall analysis of luciferase showed very high staining in the epidermis and in the hindbrain. With regard to the other organs, the staining appeared to follow a gradient: highest in ectoderm-derived organs and lowest in endodermal organs.

BLI, enabling the measurement of the reporter activity in living animals, is extremely useful for the *spatio*-temporal study of a given molecular event in single individuals; however, the two dimensional nature of the BLI outcome does not allow the exact, quantitative localization of the organ responsible for photon emission, as the deeper an organ is, the more attenuated the BLI signal, and the photon emission measured in a specific surface represents the sum of all signals coming from the different layers of tissue (Maggi & Ciana 2005). Thus, to better define the organs where ERs were most active, we carried out a series of enzymatic assays in selected tissue extracts. The measured luciferase activity changed significantly in the different organs and ranged from 10 RLU/µg protein to 4000 RLU/µg protein. In line with what was suggested by prior immunohistochemistry analyses, we found a strict correlation between the germ layer and the amount of luciferase activity (Fig. 3). The highest expression of the reporter was found in ectoderm-derived organs (brain and skin), while in the endoderm-derived organs (liver, lung, thymus and intestine), ER activity was the lowest. Mesodermic organs (heart, femur, gonad and kidney) appeared to have an intermediate concentration of the enzyme. Supplementary Figure 1 (see section on supplementary data given at the end of this article) shows that in the skin, hindbrain and forebrain, luciferase is, in general, statistically more expressed than in the other tissues.

Next, we stratified the data in relation to sex (Fig. 4). No major differences were found prior to birth. The only exception was represented by the gonads at 18.5 dpc, where females had more luciferase than males, an effect possibly associated with the very high expression of ERα protein in the ovary (Nielsen *et al.* 2000) at this embryonal stage. At P1, there was a generalized increase in luciferase activity in most organs. In the liver, bone, gonads and hindbrain, the increase observed in luciferase expression was significantly higher in males.

This result was consistent with the fact that male, not female, gonads synthesize steroids just prior to and after birth and that the testosterone synthesized is aromatized to E2 in several organs (Jones *et al.* 2000). When we crossed the ERE-Luc with the aromatase KO mice, we failed to see such an effect in male livers and femurs as well as the testis and skin of pups at P1 (Fig. 5). In the ArKOxERE-Luc mice, the luciferase activity was comparable with the levels recorded through previous foetal stages (Fig. 3; RLU/µg protein: liver 5.67±1.33; femur 42.67±9.28; testis 6.67±3.67; skin 572±75.50), which provided further

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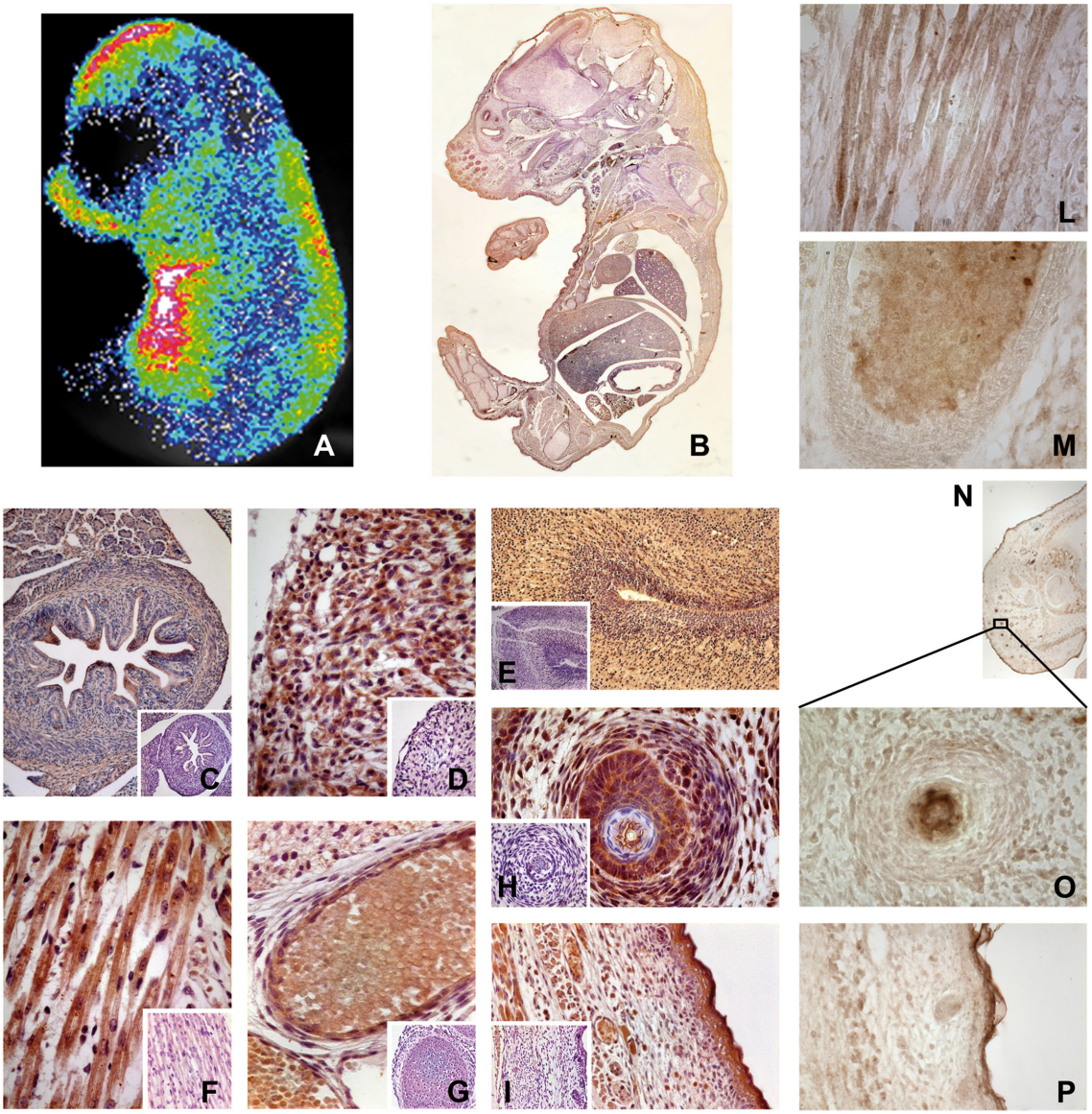

Figure 2
Immunostaining reveals ER activity in different tissues. A 17.5 dpc litter was subjected both to BLI and to IHC against luciferase and ERα. (A) Representative BLI of one mouse (signal was arithmetically increased with respect to Fig. 1B) and one of its whole-mount sections stained for luciferase (B). (C, D, E, F, G, H and I) IHC against luciferase; organ pictures were taken at 100–400× magnification: intestine (C; 100×), heart (D; 400×), hindbrain (E; 100×), muscle (F; 400×), rib (G; 400×), whisker follicle (H; 400×), skin (I; 200×). Small panels show the antibody specificity tested on a WT mouse. (L, M, N, O and P) IHC against ERα; organ pictures were taken at 100–400× magnification: muscle (L; 400×), rib (M; 400×), nose (N; reconstruction of 50×), whisker follicle (O; 400×), skin (P; 200×).

support for the hypothesis that the increased activity of the reporter observed in males only at P1 was induced by the oestrogens derived from the surge of testosterone production by male gonads (Clarkson & Herbison 2016). In addition, the observation of a basal level of ER activity in the different organs of ArKOxERE-Luc mice suggested that the unliganded ERs could be activated by factors other than circulating oestrogens.

Discussion

The present study demonstrates that, in the course of embryogenesis, ER transcriptional activity is not restricted to the reproductive organs. Indeed, ERs appear to be particularly active in tissues originating from the ectoderm, such as the brain and skin and have a lower yet still significant action in tissues, such as the gonads,

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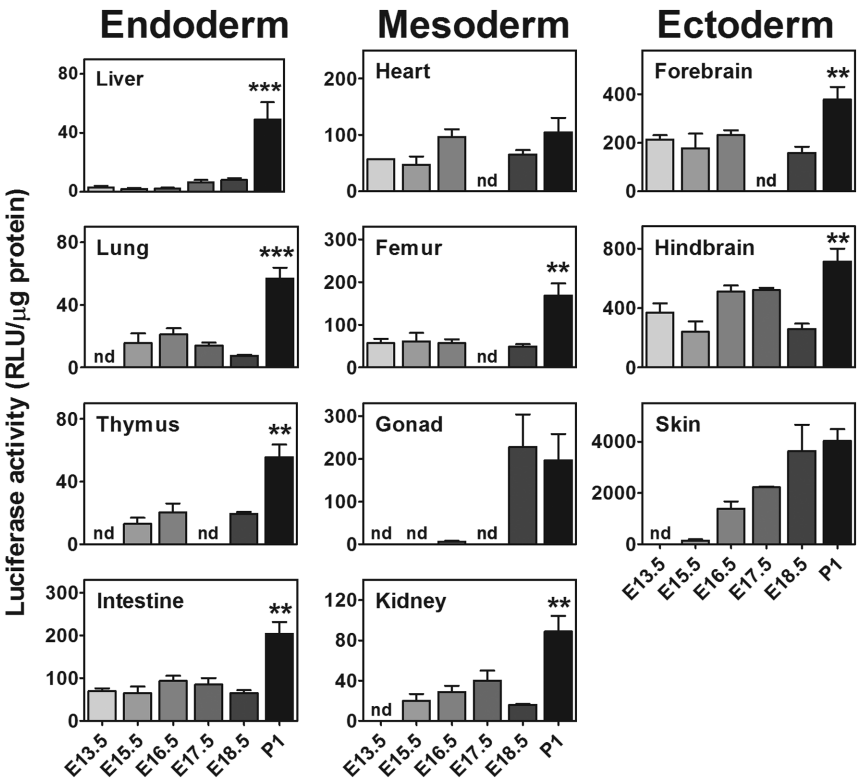

Figure 3
Foetal ER activity shows lineage-correlation. A pattern of ER transcriptional activity in different organs was obtained by measuring the luciferase activity on tissue lysates (enzymatic assay) obtained from the foetuses of both sexes. Each column contains organs from different germ layers. Bars represent the mean ± S.E.M. ($n = 6$–10 foetuses). *$P < 0.05$, **$P < 0.01$ vs the previous stage calculated by one-way ANOVA followed by the Bonferroni *post hoc* test. nd, not measured.

kidney, bone and hearth, deriving from the mesoderm. Quite interesting is the fact that ERs are minimally active in endodermic tissues, such as the lung, liver, thymus and intestine.

The mechanisms associated with this differential pattern of ER activity in the organs studied in relation to the germ-layer of origin remain to be defined. An initial explanation could be associated with a differential, tissue-specific expression of the two isoforms of ER. The lack of a detailed localization study describing the relative content of ERα and ERβ in embryogenesis does not allow us to define whether the differences of ER activity observed are due to a germ-layer-specific expression of each ER or to changes in their relative concentration with consequent potential homo- or hetero-dimerization. A second potential explanation resides in ligand availability, as it is conceivable that, depending of the stage of development, the oestrogens produced may have differential ability to reach the organs deriving from each of the three germ layers. The main source of steroid hormones during pregnancy is the maternal-placental-foetal unit (MPF unit) (Becker 2001). Measurements of oestrogen concentration in maternal blood showed a steady increase throughout pregnancy, with a peak at 17.5 dpc (Barkley *et al.* 1977); in the foetus, the peak of oestrogen synthesis is at 12.5 dpc (Lemmen *et al.* 2002). However, to be transported by the plasma, these lipophilic molecules associate with the alpha-fetoprotein (AFP), and it has been hypothesized that the high concentration of AFP produced may sequester most of the steroids produced by the MPF and may protect the foetus from the effects of maternal steroid hormones (Savu *et al.* 1981, Mendel 1989, Bakker *et al.* 2006, De Mees *et al.* 2006). Our data clearly show a germ layer-dependent ER transcriptional activity, which that could be due to either the existence of a tissue-specific uptake of circulating oestrogens or to ER unliganded activation. Indeed, endocytic pathways were described as necessary to carry steroids inside the cells (Hammes *et al.* 2005), but we still do not know whether such transport proteins are present in the embryo and are localized differentially in the various tissues. Alternatively, ERs may be differentially activated by means of a localized pattern of expression of genes encoding factors known to be able to activate these NRs in a unliganded way (such as EGF, IGF or others) (Bondy *et al.* 1990, Partanen 1990).

Regardless of the mechanisms involved, the number of tissues where ER is transcriptionally active in the course of development is suggestive of a significant biological function of the hormone–receptor complex in both reproductive and non-reproductive organs. Unfortunately, the developmental effects of the deletion of genes encoding ERα and ERβ or both isoforms have been only slightly investigated. This lack of study is possibly due the fact that none of the initial studies

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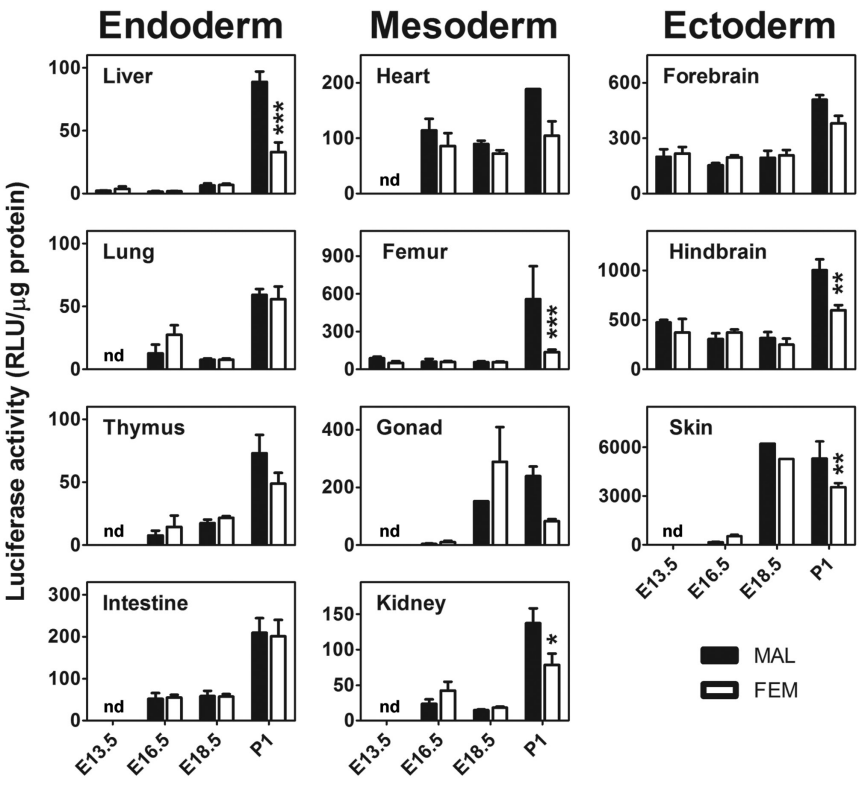

Figure 4
Gender specificity in ER activity during development. The same data shown in Fig. 3 (luciferase enzymatic assay) were stratified for sex. Bars represent the mean ± S.E.M. (n = 4–6). *P<0.05, **P<0.01 compared with opposite gender calculated with unpaired Student's t test. nd, not measured.

described major phenotypes, and both males and females survived to adulthood (Lubahn *et al.* 1993, Krege *et al.* 1998, Couse *et al.* 1999), even with fertility problems, which is particularly relevant in the ERα-null mice (Krege *et al.* 1998, Dupont *et al.* 2000, Walker & Korach 2004). However, more recent studies carried out in adults demonstrated that the deletion of ERα or ERβ is associated with malfunctions of several organs considered to be non-essential for reproduction, such as the lung, liver, adipose tissue, heart, kidney, thymus, bone and skin (Brandenberger *et al.* 1997, Lemmen *et al.* 1999, Takeyama *et al.* 2001, Walker & Korach 2004, Zoller & Kersh 2006, Carey *et al.* 2007, Foryst-Ludwig *et al.* 2008, Barros & Gustafsson 2011, Kummer *et al.* 2011, Markiewicz *et al.* 2013, Lapid *et al.* 2014, Del Principe *et al.* 2015, Tait *et al.* 2015). In the perinatal mouse brain, the *spatio*-temporal expression of ERα and ERβ was reported to contribute to organize sex differences that are not associated with

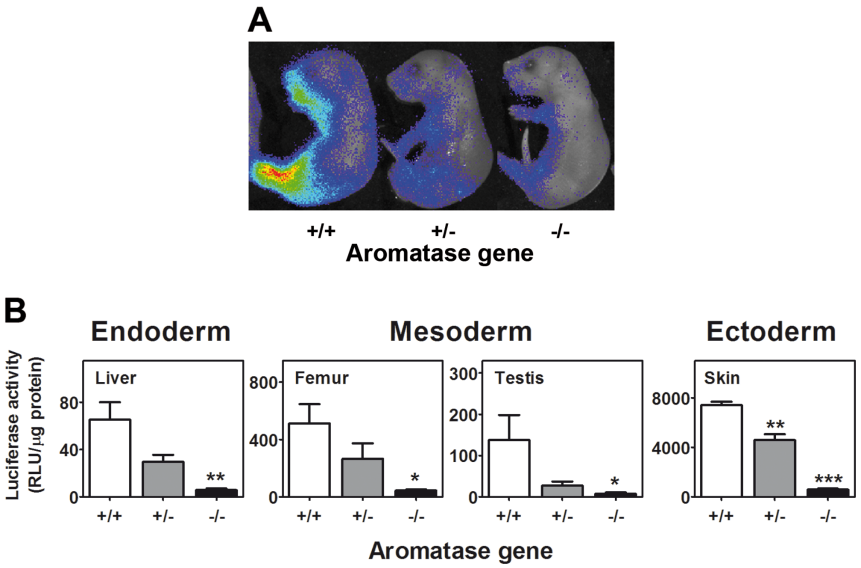

Figure 5
Lack of aromatase activity regulates ER activity in males. ERE-Luc mice were bred with Ar+/– mice, and the progeny was backcrossed to obtain the same litter Ar–/– and Ar+/+ × ERE-Luc mice. (A) Newborn P1 mice were subjected to BLI. (B) Luciferase activity was measured in tissue lysates (enzymatic assay) in which gender difference was previously observed (Fig. 4). Bars represent the mean ± S.E.M. (n = 4–6 foetuses). *P<0.05, **P<0.01 vs Ar+/+ × ERE-Luc calculated by one-way ANOVA followed by Bonferroni *post hoc* test.

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reproduction, such as the stress response and cognition (Mogi *et al.* 2015).

It is conceivable that the sexual dimorphism observed in the prevalence, course and severity of many common diseases, including cardiovascular diseases, autoimmune diseases and asthma, may originate from a sex-specific genetic architecture that is created in the course of development (also through ER activity) and that results in a male and female differential endocrine susceptibility and gene regulation, particularly in sex steroid-responsive genes. The relevance of oestrogen programming mechanisms within the mammalian foetus and perinatal time period is underlined by studies on liver metabolism that are strictly regulated by sex and where the expression and activation of hepatic ER in the course of embryo maturation and in adult life plays a prominent role (Della Torre *et al.* 2018) on energy metabolism (Yuchi *et al.* 2015, Maniu *et al.* 2016), gastric functions (Campbell-Thompson *et al.* 2001), adrenal and renal activity (Walker *et al.* 2009, Inamdar *et al.* 2015), cardiovascular activity (Del Principe *et al.* 2015) and immune functions (Zoller & Kersh 2006).

In conclusion, the results from this study and those mentioned above point to a substantial relevance of ER signalling for the correct development of mammalian embryos, and they highlight the necessity to increase our knowledge with more systematic studies aimed at understanding the necessity of these hormones, particularly for the correct development and functioning of non-reproductive organs. Such studies should not be confined to prenatal development and pups, but should be extended to adults, possibly challenged with appropriate stimuli, enabling us to test their ability to respond to alimentary and environmental stimuli, with a particular emphasis onto endocrine disrupters known to preferentially act through ERs.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/JOE-18-0003.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S D T carried out all the final analyses and control experiments. G R carried out most of the experimental work, together with L O. C M performed the immunostaining experiments. P C contributed in conceiving the study and shared his expertise on the use of ERE-Luc mice. A M conceived the project, wrote the manuscript and supervised the entire project.

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